EFFECT OF SECONDARY INJECTIONS OF ANTIGEN UPON THE RETENTION IN LIVER OF A PRIMARY INJECTION*

By JUSTINE S. GARVEY, PH.D., AND DAN H. CAMPBELL, PH.D.

(From the Division of Chemistry and Chemical Engineering, The California Institute of Technology, Pasadena)‡

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In previously published work (1, 2) it was noted that the retention of sulfanilic S^{35}-labelled hemocyanin or bovine serum albumin in liver tissue, was less, following a series of intravenous injections, than when the same amount was given in a single injection. This decrease in retention occurred in spite of the fact that immediate localization of antigen in the liver following intravenous injection, increased with repeated injections and immunization (1). For example, when rabbits were given a single injection of 50 mg. of S^{35}-labelled hemocyanin, 0.16 per cent or about 0.08 mg. was present in the liver 130 days later. When a total of 90 mg. was given in 9 injections of 10 mg. each over a period of 3 weeks an average of only 0.04 per cent or 0.04 mg. was present in liver tissue 130 days after the last injection. Essentially the same results were obtained with preparations of labelled bovine serum albumin except that the loss from liver tissue was more rapid than for the labelled hemocyanin. The question immediately arises whether multiple injections of antigen stimulate a more rapid destruction and loss of antigen deposited in tissue following the initial injection, or whether the antigen introduced in subsequent injections is more rapidly destroyed. The following investigation was, therefore, undertaken to study this reaction more thoroughly by giving rabbits an initial injection of sulfanilic S^{35}-labelled antigen followed by one or more injections of sulfanilic non-labelled antigen. By measuring the radioactivity of the liver tissue, it was then possible to determine the retention of the primary injection of antigen, and the effect of subsequent injections of antigen and circulating antibody on the retention of the primary deposition of antigen.

EXPERIMENTAL

Antigens: Hemocyanin (KLH)[1] and bovine serum albumin (BSA)[2] were both studied as sulfanilic–azo-antigens. Labelled sulfanilic acid was prepared by sulfonation of aniline with

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[1] The hemocyanin was prepared as described previously (3), by differential centrifugation of the body fluid of the giant keyhole-limpet (*Megathura crenulata*). The hemocyanin, with a molecular weight of about 6,000,000 at pH 6.0 can be centrifuged out of solution in a fairly pure state at 100,000 *g* in about 45 minutes.

[2] The BSA was supplied by Armour and Co.

S^{35}-labelled[3] and unlabelled sulfuric acid using the baking process described by Groggins (4). The diazonium salt of sulfanilic acid was prepared in the usual manner and then coupled to the native protein at pH 7.5 to 8.0 and 4°C. to form labelled azohemocyanin, S^{35}-KLH, or labelled azo-bovine-serum-albumin, S^{35}-BSA. Non-labelled azohemocyanin, S-KLH, or non-labelled azo-bovine-serum-albumin, S-BSA, were prepared in the same manner, without the use of labelled sulfanilic acid. Purification of azohemocyanin (S-KLH or S^{35}-KLH) was accomplished by repeated precipitation with 50 per cent saturated ammonium sulfate at pH 8.5, followed by solution in 0.9 per cent pyrogen-free NaCl and dialysis until free of sulfate. Azo-bovine-serum-albumin (S-BSA or S^{35}-BSA) was purified in the same manner except 60 per cent ammonium sulfate was used for precipitation.

The S-KLH and S^{35}-KLH had essentially the same properties as described previously (2). Coupling with the diazonium salt produced some degradation so that the final product showed considerable heterogeneity with respect to electrophoretic properties and molecular size. The S^{35}-KLH preparation contained an average of 80 sulfanilic acid groups per molecule of protein with a specific radioactivity of 3 to 4 mc./gm. immediately after preparation. The BSA remained fairly homogeneous after the reaction with the diazonium salt and analysis gave a value of about 35 sulfanilic acid groups per molecule of S^{35}-BSA with a specific radioactivity, immediately after preparation, of 5 to 6 mc./gm.

General Methods.—Antigen solutions were always administered intravenously into rabbits weighing 2 to 3 kilograms. Only the first injection contained labelled antigen (either S^{35}-KLH or S^{35}-BSA). Subsequent 1 ml. doses of 9 to 10 mg. of the same non-radioactive protein were administered on alternate days for a period of 3 weeks or for a shorter time as indicated in certain groups. Controls consisted of those groups which received only the primary injection.

Serums were analyzed for precipitating antibody by the addition of varying dilutions of either S-KLH or S-BSA antigen to equal amounts of antiserum. The tests were refrigerated for 48 hours; then the precipitates were centrifuged and washed 3 times with cold saline after which nitrogen was determined by the Nessler technique as described by Lanni and Campbell (5). Determination of radioactivity in liver tissue was performed as previously described in (3) using an end window flow-counter and automatic sample changer.

RESULTS

A. Retention of a Primary Injection of S^{35}-KLH, after 8 Subsequent Injections of S-KLH.—

Seventeen rabbits were injected intravenously with 2 ml. of 1.0 per cent S^{35}-KLH in 0.9 per cent NaCl. Four of these received no further injections and represented the control group, whereas, the remaining 13 were given 8 subsequent injections of 1 ml. S-KLH on alternate days. Three days after the last injection, *i.e.*, 21 days after the primary injection of S^{35}-KLH, all animals were exsanguinated by cardiac puncture and the livers were removed and perfused immediately. Serums were titrated for precipitins against S-KLH, and livers were sampled for radioactivity. The 4 control animals which had received only the radioactive injection were also killed at the same time; the serums and livers were sampled similarly as from the other 13 animals.

The 4 control animals which received only the single injection of antigen failed to show any circulating antibody by interfacial tests with antigen. The total amount of S^{35} retained in the livers of these animals was 3.9 to 4.4 per cent of the amount injected. The 13 animals which received a similar amount of

[3] The S^{35}-labelled sulfuric acid was obtained in carrier-free form from the Oak Ridge National Laboratories.

S^{35}-labelled antigen in a primary injection, and in addition a series of injections of non-radioactive antigen, had a wide variation in the retention of the initial injection as shown in Fig. 1 in which the retention of S^{35} has been presented with relation to concentration of antibody. It will be seen that there was an inverse

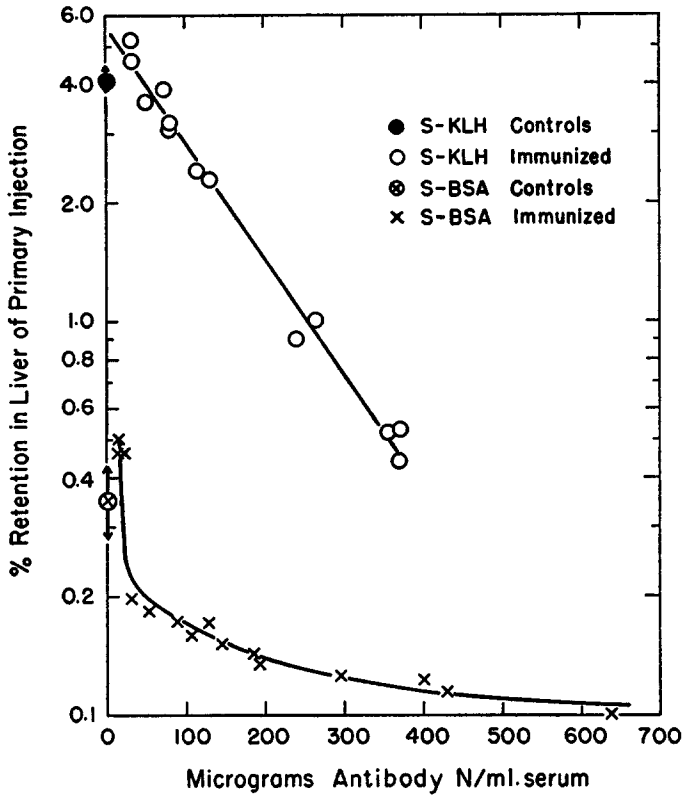

FIG. 1. A semilog plot of retention *versus* circulating antibody titer. The retention is from a primary injection of either S^{35}-labelled hemocyanin (S^{35}-KLH) or bovine serum albumin (S^{35}-BSA) when followed by 8 injections of the same but non-radioactive antigen. For controls, the center of a circle indicates the mean of the distribution which is shown by arrows. Other points are for a single, immunized animal. All data were obtained at 3 days after the last injection in the series of 8 non-radioactive injections.

relation between retention of antigen from the primary injection and the amount of circulating antibody resulting from secondary responses.

B. Retention of a Primary Injection of S^{35}-BSA after 8 Subsequent Injections of S-BSA.—

Each of 15 rabbits was injected intravenously with 2 ml. of 0.7 per cent S^{35}-BSA, followed by eight 1.0 ml. injections of 1.0 per cent S-BSA given on alternate days. Three days after

the last injection, *i.e.* 21 days after the primary injection of S^{35}-BSA, the animals were exsanguinated by cardiac puncture. The livers were removed, perfused with 0.9 per cent NaCl, weighed and sampled in triplicate for radioactivity. Serums were analyzed quantitatively for precipitating antibody against S-BSA.

Injected simultaneously with the group of 15 rabbits was another group of 8 rabbits which received only the radioactive injection. The liver tissue from this once-injected group was

TABLE I

Liver Retention of a Primary Injection of S^{35}-KLH Compared to Retention of the Same Injection When Followed by Subsequent Non-Radioactive Injections

Rabbit No.	Time after the primary injection of S^{35}-KLH	Immunized			Controls
		No. of S-KLH Injections	μg. of antibody N per ml. of serum*	Per cent‡ of S^{35}-KLH retained in liver	Per cent of S^{35}-KLH retained in liver
	days				
—	0.25				32.5
—	3				9.9
10-12	5	1	2	8.6	7.6
10-13			2	8.2	
10-15	7	2	2	6.4	6.2
10-16			2	6.5	
10-18	10	3	81	0.9	4.5
10-19			13	4.0	
10-21	12	4	180	2.0	—
10-22			59	3.5	
10-24	14	5	89	3.1	3.2
10-25			106	3.2	
10-27	17	6	73	3.0	—
10-28			62	2.8	
10-30	19	7	102	2.7	—
10-31			297	1.1	
10-33	21	8	284	1.0	2.0
10-34			305	0.9	
—	27				1.5
—	35				1.2

—Indicates no data for one group of animals at an interval of time for which there were data on animals of the other group.

* The control animals failed to show any circulating antibody.

‡ Per cent of total amount injected.

sampled for radioactivity at the same time as the multiple-injected group so that the number of days of S^{35} decay was the same in tissues of both groups.

As shown in Fig. 1, the once-injected group had higher retention (0.28 to 0.43 per cent) than the multiple-injected group except for 3 animals in the latter group which were extremely low in antibody titer. Although the retention of S^{35}-BSA was at a much lower level than with the S^{35}-KLH antigen, there was a similar inverse relation between retention of the antigen from the primary injection and the circulating antibody resulting from secondary responses.

C. Retention of a Primary Injection of S^{35}-KLH When Followed by from 1 to 8 Subsequent Injections of S-KLH.—

A control group of 9 rabbits received a single intravenous injection of 20 mg. of S^{35}-KLH. They were sacrificed at 9 different times (from 6 hours to 35 days later), and liver tissue was analyzed for S^{35} as described previously (3). Tests for circulating antibody were consistently negative in all animals. A second group of 16 rabbits also received a primary intravenous injection of 20 mg. of S^{35}-KLH and then a varying number of subsequent 10 mg. injections of S-KLH at intervals of 2 to 3 days. Two rabbits were sacrificed 3 days after each subsequent injection of S-KLH; livers were analyzed for S^{35} and serums for antibody against S-KLH.

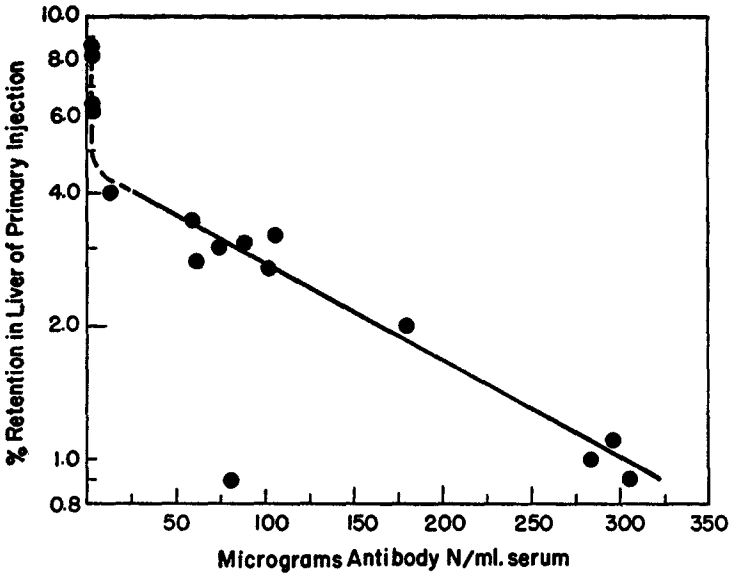

FIG. 2. A semilog plot of retention *versus* circulating antibody titer drawn from data in Table I. The amount of antigen varied from 1 to 8 non-radioactive injections following the primary radioactive injection for which retention is given.

The results of this experiment have been presented in Table I. The control group showed a consistent loss of S^{35} from livers which was relatively rapid during the first 2 to 3 weeks and then tended to level off at the value of 1.0 per cent of injected material at 4 weeks. (This is essentially the same as described previously (1) where a semilog plot of retention against time gives a fairly smooth curve.)

The other group of animals which received subsequent injections of S-KLH showed a great deal of variation. Although there was some tendency for an inverse relation between amount of circulating antibody and per cent retention this was not always consistent. For example, in the initial time intervals of 5 and 7 days when antibody began to appear in the circulation, the retention

was slightly higher than in the controls. This effect has also been noted in other similar experiments. It will be noted also in Fig. 1 that retention in low titer animals (both S-KLH and S-BSA groups) was slightly higher than the once-injected controls. The same effect was also noted in experiments in which attempts were made to modify retention by passive transfer of homologous antibody (see below). Of the remaining 12 rabbits, 5 (Nos. 10-18, 10-21, 10-31, 10-33 and 10-34) showed a marked deviation in retention from the corresponding control animal. In each of these instances the titer of circulating antibody was relatively high for the corresponding period of immunization.

Fig. 2 is a semilog plot of the retention *versus* circulating antibody. There is a similar relationship to that found in Fig. 1 in spite of the fact that the number of antigen injections varied, consequently varying the time when retention of the primary injection was determined.

D. The Effect of Subsequent Injection of Homologous Antibody on Retention of Antigen.—Since it was obvious from the foregoing experiments that the loss of antigen from liver tissue was proportional to the amount of circulating antibody in actively immunized animals, the following experiment was carried out to determine whether this effect were directly related to circulating antibody or to some more subtle intracellular factors involved in the production of antibody.

Fourteen rabbits received 2.0 ml. S^{35}-KLH solution which contained about 15 mg. of protein. Eight days later, 5 animals received rabbit gamma globulin containing antibody against S-KLH and 9 controls received a similar amount of gamma globulin from normal rabbits. The gamma globulin was prepared from pooled serum by ammonium sulfate precipitation as described previously (1).

Another group of 13 rabbits received 2.0 ml. of S^{35}-BSA solution which contained about 15 mg. of protein. Eight days later, 7 received rabbit gamma globulin containing antibody against S-BSA and 6 controls received a similar amount of gamma globulin from normal rabbits.

The amount of antibody injected was determined by a precipitation test with the same antigen which had been injected. The actual amount of circulating antibody was studied by precipitation tests with serum obtained from a 10 minute bleeding after the antibody injection. Precipitating antibody was studied at later intervals of time, dependent upon the time chosen for the liver retention determination. Animals were killed at one of four time intervals after antibody injection, namely 6 hours, 1 day, 3 days, and 7 days. The radioactivity was determined in the liver tissue in the usual manner and the results are given in Table II together with data obtained from N determinations for total protein injected and for precipitating antibody.

This experiment, as well as two previous similar experiments, failed to show that passively transferred antibody had any consistent effect on retention of antigen in the liver.

E. Effect of Secondary Injections of a Non-Cross Reacting Non-Radioactive Antigen on Retention of a Primary Injection of Radioactive Antigen.—The following experiment was carried out to determine whether the effect of antibody formation on retention of antigen were a specific mechanism.

TABLE II

Effect of Passive, Homologous Antibody on Liver Retention of a Primary Antigen Injection Given 8 Days Earlier

Time after Ab injection	Animal No.	Ag	Gamma globulin injection				Ag retention	Variation from control	μg. Ab N assayed /ml. serum				
			Kind	Injected vol.	Total protein	Ab protein injected			10 min.	6 hrs.	1 day	3 days	7 days
				ml.	*gm.*	*mg.*	*per cent*	*per cent*					
6 hrs.	8-11	S^{35}-KLH	Normal	10.0	0.71	—	5.30						
"	8-10	"	S-KLH	"	0.70	348	4.27	−20	190	115			
6 hrs.	9-77	S^{35}-KLH	Normal	15.0	1.05	—	5.38						
"	9-83	"	"	"	1.05	—	5.47						
"	9-81	"	S-KLH	"	1.07	391	5.12	−5	141	77			
1 day	9-84	S^{35}-KLH	Normal	10.0	0.71	—	4.84						
3 days	8-13	S^{35}-KLH	Normal	10.0	0.71	—	3.54						
"	8-12	"	S-KLH	"	0.70	348	4.42	+20	130		111	69	
3 days	9-78	S^{35}-KLH	Normal	11.0	0.71	—	3.26						
"	9-85	"	"	"	0.71	—	4.98						
"	9-80	"	S-KLH	"	0.78	287	3.78	+14 −24*	115		38		
7 days	9-79	S^{35}-KLH	Normal	9.0	0.63	—	2.62						
"	9-86	"	"	"	0.63	—	2.80						
"	9-82	"	S-KLH	"	0.64	235	2.16	−20	89		30		
6 hrs.	8-17	S^{35}-BSA	Normal	10.0	0.68	—	0.81						
"	8-14	"	S-BSA	"	0.60	194	0.96	+19	107	75			
6 hrs.	9-73	S^{35}-BSA	Normal	10.0	0.70	—	0.71						
"	9-67	"	S-BSA	"	0.75	394	0.92	+30	250	132			
"	9-71	"	"	9.5	0.71	380	0.53	−25	193	104			
1 day	9-74	S^{35}-BSA	Normal	10.0	0.70	—	0.84						
"	9-68	"	S-BSA	"	0.75	394	0.68	−19	276		112		
3 days	8-15	S^{35}-BSA	Normal	10.0	0.68	—	0.56						
"	8-16	"	S-BSA	"	0.60	194	0.64	+14	112		85	57	
3 days	9-75	S^{35}-BSA	Normal	10.0	0.70	—	0.47						
"	9-69	"	S-BSA	"	0.75	394	0.35	−26	334		108	74	
7 days	9-76	S^{35}-BSA	Normal	10.0	0.70	—	0.24						
"	9-70	"	S-BSA	"	0.75	394	0.23	−4	194		79	49	42

Ab, antibody.
Ag, antigen.
* With comparison to varying values for the control.

Ten rabbits received an intravenous injection of 2 ml. of 0.75 per cent S^{35}-KLH in saline. Of these, 4 received no further injections. The remaining 6 animals received 8 additional injections of 1.0 per cent BSA as 1.0 ml. doses at intervals of 2 to 3 days. Three days after the last injection of BSA, i.e. 21 days after the injection of S^{35}-KLH, all 10 animals were killed.

At this time the radioactivity of the liver tissue was essentially no different in animals which received the secondary injections and those which served as

controls with only the radioactive injection. The control animals which received only the initial injection of S^{35}-KLH gave an average retention of 2.1 per cent. Those which received 8 additional injections of BSA gave an average retention of 2.0 per cent.

DISCUSSION

We speculated at one time (1) that the increased rate of loss of a primary injection of antigen as a result of subsequent injections of the same antigen, might be the result of establishing new equilibrium conditions in which some of the primary antigen was replaced by some of the secondary antigen. However, the data obtained failed to show any relationship between rate of loss of the primary antigen and the number of secondary injections except from the standpoint of antibody production. This correlation with antibody formation rather than with the number of antigen injections is clearly shown in Table I and Fig. 2. The one exception was animal No. 10-18 which received 3 secondary injections of S-KLH and showed an antibody level of 81 μg./ml. of serum and a low retention of antigen of only 0.93 per cent. One can only speculate that had this animal been allowed to survive, it would have shown an unusually high antibody-forming activity and thus the antibody level was unusually high for such an early period of immunization. It was of interest that this animal also produced an abnormally high excretion of antigen following the third injection of S-KLH. The relationship between retention of a primary injection of antigen and immunization to the same antigen was noted with both hemocyanin and bovine serum albumin, although as noted previously (2), the hemocyanin antigen was retained to a greater extent, stimulated a greater antibody response and was excreted at a slower rate than the serum albumin.

The relation of circulating antibody to retention of antigen stored in liver tissue is still not clear, but the experiments on active immunization leave no doubt that there exists an inverse relation between antibody and antigen retention. Since our experiments consistently failed to show that passive transfer of antibody had any significant effect on the rate of loss of antigen from the liver, we must conclude that stored antigen is influenced by some mechanism which involves an intracellular immune mechanism and not antibody in the blood stream. Circulating antibody in this system is only an indirect measure of antibody-forming activity and is not per se the factor responsible for the catabolism or loss of antigen from liver tissue.

Dixon (6) concluded from the results obtained by him and his collaborators, that the increased catabolism of I^{131}-labelled antigen was apparently dependent only upon circulating antibody and that there was no evidence suggesting any involvement of cells. These conclusions are entirely justified from their experimental data, but their experimental system was much different from ours. For example, their results (7) were based first upon the injection of antigen into immunized animals and second

upon the excretion of urine or elimination from the blood stream of I^{131}. Our investigation deals primarily with the fate of, and effect of antibody formation on, antigen deposited in the liver of normal rabbits.

From experiments with tissues, McMaster *et al.* (8) concluded that if a species (such as the rabbit) gives a good antibody response to a specific antigen, the antigen is not retained long. Conversely, if little antibody is formed, as in the mouse, retention may be prolonged in the blood and liver. Although blood only was studied for antigen retention, Johnson *et al.* (9) made a suggestion along the same line, *i.e.* "that poor antibody formers are the result of the inability to rid antigen from tissues." However, the actual elimination of antigen may not be the important factor involved in antibody formation; the breakdown and subsequent elimination of antigen may be merely an indication of antibody-forming activity going on within the cell.

The question still remains whether the liver plays any role in antibody formation, and if so, what part. However, there is little doubt that in some way intracellular antibody-forming activity influences the loss (or retention) of antigen deposited in liver tissue and perhaps other tissues.

It is also significant that the effect of immunization on the retention of antigen is of a specific nature, which may eventually give a clue as to the mechanism of antibody formation.

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SUMMARY

The retention of antigen in rabbit liver tissue, resulting from a primary intravenous injection, is influenced by immunization brought about by subsequent intravenous injections of the same antigen. In rabbits given a single primary intravenous injection of radioactive antigen, the retention of radioactivity in liver tissue, after a period of 21 days, was greater than when the primary injection was followed by secondary injections of the same, but non-radioactive antigen. The results were similar for both S^{35}-azohemocyanin and S^{35}–azo–bovine-serum-albumin, except the hemocyanin was retained to a greater extent than the albumin.

There was very little if any correlation between the number of secondary injections and retention of the initial injection. Quantitative antibody nitrogen data, obtained for the serum of each rabbit showed, in general, an inverse relationship between circulating antibody and radioactivity retained, *i.e.* the higher the circulating antibody titer, the lower the retention of radioactivity in liver tissue.

Passively administered homologous antibody did not produce a change in the retention of the primary injection of antigen nor did secondary injections of a heterologous native protein injected according to the same immunization schedule as the homologous azoprotein. From these results it may be concluded

that an intracellular antibody-forming activity influences the loss (or retention) of antigen deposited in liver tissue and that the mechanism is immunologically specific.

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